Bacillus thuringiensis-toxin resistance management: Stable isotope assessment of alternate host use by *Helicoverpa zea*

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Data have been lacking on the proportion of *Helicovera zea* larvae that develop on noncotton host plants that can serve as a refuge from selection pressure for adaptation to transgenic cotton varieties that produce a toxin from the bacterium *Bacillus thuringiensis*. We found that individual *H. zea* moths that develop as larvae on cotton and other plants with C_3 physiology have a different ratio of ^{13}C to ^{12}C than moths that develop on plants with C_4 physiology, such as corn. We used this finding in determining the minimum percentage of moths that developed on noncotton hosts in two cotton-growing areas. Our results indicate that local corn can serve as a refuge for *H. zea* in midsummer. Our results contrast dramatically with the prevailing hypothesis that the large majority of late-season moths are produced from larvae feeding on cotton, soybean, and other C_3 plants. Typically, <50% of moths captured in August through October have isotope ratios indicative of larval feeding on C_3 plants. In one October sample, 100% of the moths originated from C_4 hosts even though C_4 crops were harvested at least 1 mo earlier, and no common wild C_4 hosts were available. These findings support other research indicating that many late-season *H. zea* moths captured in Louisiana and Texas are migrants whose larvae developed on corn in more northern locations. Our isotope data on moths collected in Texas early in the season indicate that the majority of overwintering *H. zea* do not originate from cotton-feeding larvae and may be migrants from Mexico. Non-Bt corn in Mexico and the U.S. corn belt appears to serve as an important refuge for *H. zea*.

stable carbon isotopes | cotton | corn

Since 1996, the U.S. Environmental Protection Agency (EPA) has granted a number of conditional permits for commercialization of genetically engineered cotton and corn that produce insecticidal proteins called *Bacillus thuringiensis* (Bt) toxins (1). One condition associated with most of these permits is that toxin-free plants must always be available to populations of the target insect pests. These nontoxic plants are needed to serve as a refuge for pest individuals with toxin-susceptibility genes (1–5). Genetic models and experiments have shown that such refuge plants can significantly slow the evolution of resistance to the Bt toxin, especially if the refuge plants produce 500 or more susceptible insects for every resistant insect produced in the engineered crop (2, 4–6). The 1998 EPA Science Advisory Subpanel (2) concluded that in addition to having refuges, the registered Bt crops must produce a high dose of toxin capable of killing even partially resistant insects.

Engineered cotton and corn cultivars produce a high dose of the Cry1A forms of Bt toxin relative to the toxin tolerance of some major insect pests such as *Heliothis virescens* (F.) and *Ostrinia nubilalis*, but not for *Helicoverpa zea* (commonly known as the bollworm, the corn earworm, or the tomato fruitworm), which has a naturally high tolerance for most Bt toxins (7–9). When plants do not produce a high dose of toxin for a specific pest, a very large refuge must be maintained to reach the 500:1

ratio (2, 10). For *H. zea*, it is especially critical to have a large refuge, because there is evidence of Bt resistance genes in field populations (11, 12). The risk of resistance to Bt in *H. zea* is rated as "high" and should require at least a 40% refuge according to an industry/academia report (13).

In many cotton-growing regions, Bt cotton varieties comprise >60% of all cotton planted (www.epa.gov/oppbppd1/biopesticides/reds/brad_bt_pip2.htm). In the southeast and midsouth, the major noncotton host plant for *H. zea* is thought to be corn (e.g., refs. 14 and 15). EPA initially granted permission for planting only a token amount of Bt corn in the southern cotton-growing areas so that corn could serve as a refuge for *H. zea*. EPA later allowed the planting of 50% Bt corn in these areas, which is substantially lower than the 80% that is permitted in regions where cotton is not grown. There are times in the summer when corn is expected to produce almost all of the *H. zea* moths in some cotton-growing areas (15), but for most of the season, we have lacked quantitative data on the ratio of *H. zea* produced in cotton vs. corn.

Because *H. zea* is polyphagous, other crops (e.g., sorghum, tomato, and soybean), weeds, or natural vegetation could potentially serve as refuges. There have been many claims and anecdotal reports about the contribution of plants other than non-Bt cotton and corn to the *H. zea* refuge (ref. 2 and www.epa.gov/pesticides/biopesticides/reds/brad_bt_pip2.htm), but rigorous supportive data are lacking. The EPA's currently mandated refuges for cotton and corn discuss only acreage of non-Bt cotton and corn, respectively, in determining whether a sufficient number of susceptible insects are being produced (e.g., www.epa.gov/pesticides/biopesticides/otherdocs/bt_cotton_refuge_2001.htm); however, there are some unwritten assumptions by EPA that other crops add to the refuge for *H. zea*. EPA's recent reregistration permits for Bt cotton and corn (www.epa.gov/pesticides/biopesticides/reds/brad_bt_pip2.htm) are conditioned on the provision by the registrants of quantitative data on alternate host use by *H. zea* within the next 2 yr.

Until recently, the only way to assess the relative number of *H. zea* produced outside the cotton crop has involved time-consuming field surveys that estimate how many large larvae are feeding on all other crops, weeds, and natural vegetation. The few attempts at these surveys have produced variable results with low resolution (e.g., refs. 14, 16, and 17). Another problem is that these surveys assess only larval production. The information needed by the EPA is on moth production from the refuges, and it is not always possible to accurately predict adult numbers from larval numbers because of predation, parasitism, and differential suitability of soils for pupal survival (18).

AGRICULTURAL SCIENCES

Implicit in the EPA requirement of smaller corn refuges in noncotton-growing areas is the assumption that *H. zea* moths produced from Bt corn in those areas would not contribute resistance genes to future generations. This assumption is based on the conventional wisdom that *H. zea* moths migrate to northern corn belt states from the cotton belt in the summer, but that there is no return southward migration in the fall (18, 19). Because *H. zea* is typically unable to survive the winter in northern states, the individuals in those areas are expected to perish (20).

To improve our understanding of host use and seasonal movement by *H. zea*, we have studied the stable carbon isotope ($^{13}C/^{12}C$) composition of *H. zea* moth wings. Plants possessing C_3 physiology, such as cotton, are more depleted in ^{13}C relative to ^{12}C than C_4 plants such as corn (21). The $^{13}C/^{12}C$ content, commonly reported as $\delta^{13}C$, can be affected by factors such as plant stress and geographic location, but the largest difference typically occurs between C_3 and C_4 plants (21, 22). The $\delta^{13}C$ ranges for C_3 and C_4 plants are -20 to $-32‰$ and -9 to $-17‰$, respectively (23). Archeologists have found that the $^{13}C/^{12}C$ composition of humans and other animals reflects the carbon isotopic composition of their foods (24, 25). More recently, Tallamy and Pesek (26) found that elytra of *Diabrotica* beetles reared on squash (a C_3 plant) were more depleted in ^{13}C than elytra from beetles reared on corn. Although the host of the adult influenced the isotope ratio in a beetle's elytra, the impact of the larval diet was always evident.

We hypothesized that if a moth of *H. zea* was found to have a $\delta^{13}C$ value similar to a C_4 plant, the moth did not develop on cotton. Similarly, a $\delta^{13}C$ value between -20 and $-32‰$ would indicate that the moth did not develop on corn. The stable carbon isotope technique used in our study was not expected to distinguish between insects that fed on different C_3 plants such as soybean and cotton.

Here we report on research that confirms our hypothesis by demonstrating that the wings of *H. zea* individuals that were reared on cotton and soybean have greater ^{13}C depletion than the wings of *H. zea* individuals reared on corn. Furthermore, we estimate the percentage of moths that do not develop on cotton during the field season in one area of Louisiana and in one area of Texas. These data have important implications for development of Bt resistance management programs.

Materials and Methods

***H. zea* Rearing.** In 1997 and 1998, last instar *H. zea* larvae were collected from cotton and soybean fields at the Central Crops Research Station (CCRS), located near Clayton, NC. Plant material was also collected from these fields. All samples were brought back to the North Carolina State University (NCSU) campus. Larvae were reared to pupation on field-collected leaves and fruiting structures in 250-ml plastic cups. In 1997 and 1998, corn ears at the CCRS infested with last instar larvae were picked and placed in large coolers with 2 inches of moist sand on the bottom. Larvae completed their development in the corn ears and pupated in the sand. Pupae were collected and placed individually in 30-ml plastic cups until emergence. Within 48 h of emergence, adults were placed in 95% ethanol and stored until analysis. Ethanol was removed by evaporation before analysis and did not affect the carbon isotope ratios of the wings (moths stored in ethanol mean $\delta^{13}C = -24.75‰$, SE = 0.21; moths never placed in ethanol, mean $\delta^{13}C = -24.99‰$, SE = 0.28).

***H. zea* Moth Collection.** In Louisiana, moths were collected in 1997, 1998, and 1999 from five pheromone traps, each spaced at least 0.4 km from each other. The traps were generally set up on the edges of 4-acre fields within a crop mix of cotton, soybean, sorghum, and corn, at the Red River Research Station, located in a cotton-/corn-/soybean-growing area (Bossier Parish). Up to

100 moths were collected within each 2-wk period, and typically sufficient moths were collected within the first week of a collecting period. Moths were stored in 95% ethanol. At the end of the season, all collections were sent to NCSU for analysis. Moths were collected from six pheromone traps located in the Brazos River Valley near College Station, TX in 1997, 1998, 1999, and 2000. These traps were generally checked every 2–3 d, and only live moths were collected. As above, moths were stored in ethanol and shipped to NCSU.

Sample Preparation. Moths were removed from the alcohol and were placed on paper towels to blot away excess alcohol. One forewing of each moth was clipped off and placed in an open 2.5-ml plastic vial under an air stream to evaporate the residual alcohol; the vial was then capped and stored. The remainder of the moth was placed in a separate alcohol-filled vial. Both vials were labeled in a manner that allowed individual moths and wings to be matched in case followup analyses were needed to confirm carbon isotope data obtained from the first wing.

Stable Isotope Analysis. The largest set of moth samples was analyzed at NCSU. The dried wings were placed in tin capsules and combusted to CO_2 in a Carlo Erba 1108 CHNS analyzer. The CO_2 was trapped cryogenically for isotopic analysis (27). The $\delta^{13}C$ measurements were made on a modified Finnigan-MAT (San Jose, CA) Delta E isotope ratio mass spectrometer (28). Isotopic standards were analyzed with the samples.

Samples from later dates were sent to more automated stable isotope labs at the University of Utah and the University of Georgia on a contract basis. Equipment used at the University of Utah was a Carlo Erba 1108 element analyzer coupled to a Finnigan-MAT Delta S mass spectrometer with a Finnigan-MAT Conflo II Interface. The equipment at the University of Georgia included a Carlo Erba NA 1500 CHN Analyzer coupled to a Finnigan-MAT Delta C Mass Spectrometer also using a Conflo II Interface.

To verify the consistency of the analyses between the laboratories, the second wings from 30 moths that had been analyzed at NCSU were sent to the University of Utah as blind samples. For the 25 moths in the sample with isotope ratios indicative of feeding on C_3 plants, the mean and standard deviation of the depletion value was $-26.57 \pm 1.41‰$ for wings analyzed at NCSU and $-26.55 \pm 1.53‰$ for wings analyzed at the University of Utah. For the five moths with isotope ratios indicative of C_4 plant feeding, the values for wings analyzed at NCSU were $-12.52 \pm 0.83‰$ and $-12.46 \pm 1.21‰$ for wings analyzed at the University of Utah. These extremely close values demonstrate the precision of the stable carbon isotope measurements.

The number of moth samples analyzed for each date/location is small because of the time and cost of analysis. Except for the Texas samples from 2000, we analyzed only samples from selected periods during the *H. zea* flight season.

Results

Isotopic Differentiation of Moths from C_4 and C_3 Plants. Wings from moths reared on cotton, corn, and soybeans in the laboratory had $\delta^{13}C$ values ranging from $-26.53‰$ to $-23.89‰$ ($n = 11$), $-13.77‰$ to $-12.69‰$ ($n = 9$), and $-28.27‰$ to $-27.23‰$ ($n = 5$), respectively. There was no overlap between cotton- and corn-reared moths. The complete resolution of the two plant food sources is further demonstrated in Fig. 1, where $\delta^{13}C$ values are plotted for a randomly chosen set of 325 wings from field-collected moths. Results from these analyses enabled us to classify any moth with a value of less than $-20.0‰$ as having fed on a C_3 plant, and any moth with a value of more than $-15.0‰$ as having fed on a C_4 plant. The absence of intermediate values indicates that there are no major host plants that produce moths with intermediate values. The absence of intermediate values
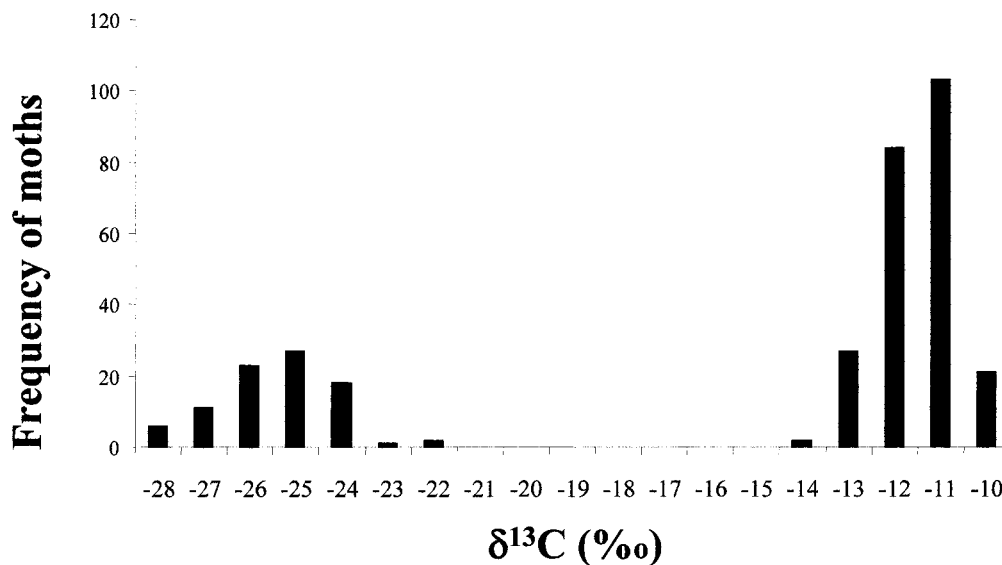
Fig. 1. The frequency of $\delta^{13}C$ values for a sample of 325 wings from field collected moths.

also indicates that an individual larva rarely fed on both corn and one of the dicot weeds that are often found in corn fields. Had adult nectar feeding on C_3 plants significantly affected the ratio of carbon isotopes in wings, this too could have resulted in intermediate values for moths that developed on C_4 plants.

Seasonal Patterns of Host Use in Louisiana. The percent of moths in 1997–1999 that were categorized as having fed on C_4 plants as larvae is depicted in Fig. 2. Early in the season, there appears to be a mixed use of C_3 and C_4 plants, which fits with expectations from larval *H. zea* surveys that indicate early season use of corn, weeds, and native hosts before cotton is mature enough to be a common host (e.g., refs. 16 and 29). Most of the recorded weed

hosts of *H. zea* are C_3 plants (16, 29, 30). In July samples, most or all *H. zea* wings have $\delta^{13}Cs$ consistent with previous larval feeding on corn. This pattern makes sense, because Louisiana corn is in its most attractive stage (31) for egg laying in June [$\approx$60% of corn has reached the attractive silking stage by June 12 (http://usda.mannlib.cornell.edu)], and development from egg to adult takes about 4 wk. Some of the C_4 moths captured in midseason may have developed on sorghum, which has C_4 physiology. However, in northwest Louisiana, sorghum acreage is about 10% that of corn (http://usda.mannlib.cornell.edu) and typically has light infestation by *H. zea* with high rates of predation and parasitism.

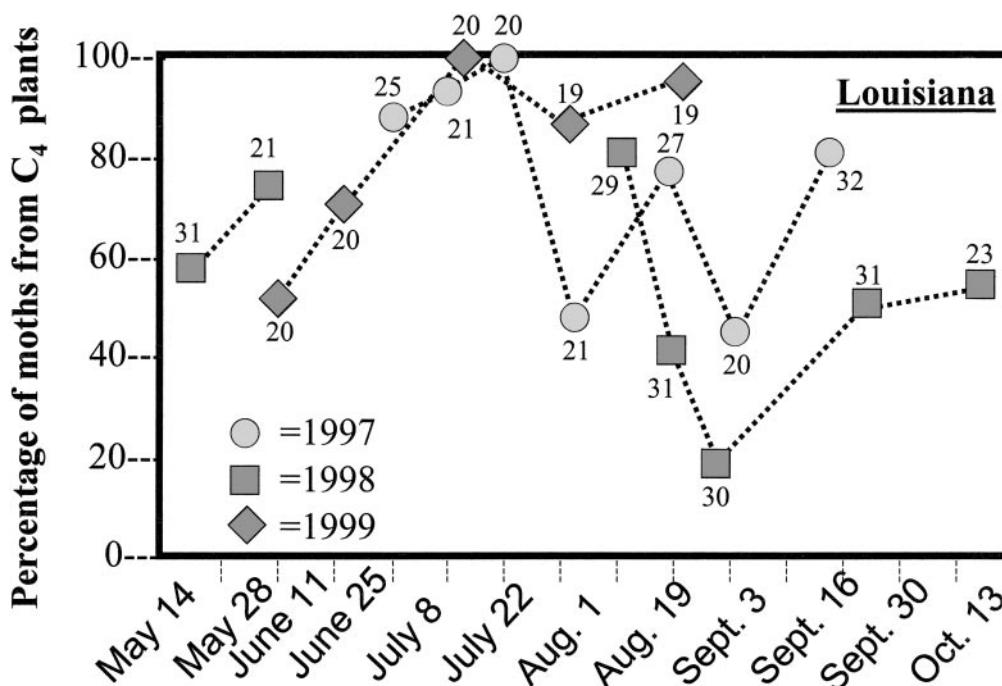In the late August through October samples, the wing isotopic

Fig. 2. Percentage of moths in Bossier Parish, LA, in 1997, 1998, and 1999 with carbon isotope ratios indicative of having fed on a C_4 plant as larvae. The number of moths from each sample that was tested is indicated next to each symbol.

AGRICULTURAL SCIENCES

Fig. 3. Percentage of moths in the College Station, TX, area in 1997, 1998, and 1999 with carbon isotope ratios indicative of having fed on a C_4 plant as larvae. The number of moths from each sample that was tested is indicated next to each symbol.
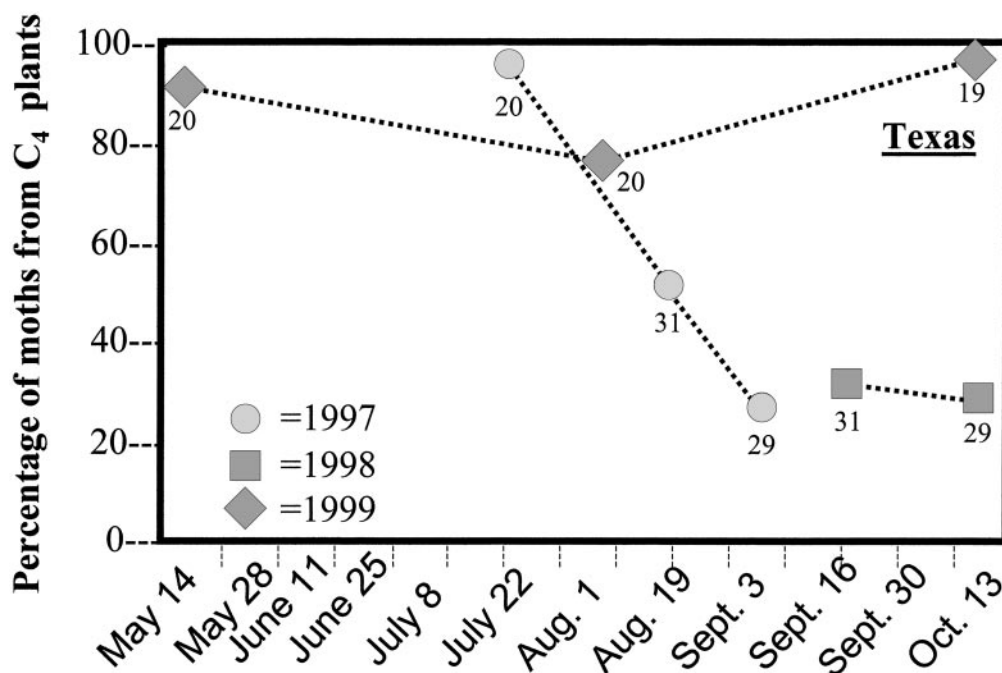
compositions do not follow the conventional wisdom that as the silks of corn mature and dry, eggs are primarily laid on cotton and soybean (14–16, 31). By July 6, over 94% of corn in Louisiana has passed the silking stage (http://usda.mannlib. cornell.edu), so moth numbers from corn should begin to decline about 4 wk later. In the August 27, 1998 sample, 83% of moths appear to have developed on C_3 plants, but in all of the other late-season samples, between 45% and 95% of the wings have $^{13}C/^{12}C$ content reflecting a C_4 host. In Louisiana, there are no major C_4 crops or weeds known to support *H. zea* growth late in the season (e.g., ref. 16). However, the existence of an unreported native C_4 host plant cannot be ruled out, because surveys have not been comprehensive and were mostly conducted decades ago. Such a C_4 host would need to be a very good host, because the number of moths captured late in the season is often high. For example, more moths were captured from September 1 until October 1, 1998, in Louisiana than at any other time during that season (www.agctr.lsu.edu/inst/research/stations/ redriver/sm/smres5.htm). It is also possible that the late-season moths with levels of ^{13}C depletion similar to C_4 plants were long-distance migrants produced from larvae that had fed on corn in more northern areas where corn matures at a later date.

Seasonal Patterns of Host Use in Texas. Because the host use in Louisiana late in the season was not consistent with previous conclusions about *H. zea* host use, we analyzed carbon isotope values for late-season moths collected near College Station, TX. In this area, as in Louisiana, corn silks in mid-June [41% silking in Texas by June 12 (http://usda.mannlib.cornell.edu)]. However, unlike the Red River Valley of Louisiana, the area sampled in Texas is very dry in the late season and supports little growth of host plants other than irrigated cotton.

Fig. 3 shows the ^{13}C categorization of *H. zea* wing samples from 1997, 1998, and 1999. In Texas, the proportion of the late-season moths coming from C_4 plants is somewhat lower than in Louisiana, but it is always above 25%. This is very surprising, given the lack of any known C_4 host plants in the surrounding area at that time. The one October sample in 1999 with 100% of

the moths coming from C_4 hosts is especially striking. As in Louisiana, the highest within-season moth captures in some years are recorded late into September and October (ref. 32 and J.L., unpublished data).

Samples from the entire 2000 season in Texas were analyzed and indicated that there were no periods during which cotton could have produced significantly more than 70% of *H. zea* moths (Fig. 4). For all years, the average percentage of moths collected between August 1 and October 20 with C_3 isotope values was only 40.4%. Some of these moths may have fed on soybean. The early-season data from March and April 2000 indicate that most of the overwintering moths must have developed on noncotton hosts.

Discussion

The results presented here offer, to our knowledge, the first quantitative assessment of seasonal *H. zea* moth production from noncotton host plants and should therefore be of use to the EPA in establishing a more data-based resistance management program. In the southern cotton-growing regions of the U.S., it has typically been assumed that cotton and soybean are the predominant hosts of *H. zea* in the latter part of the growing season because corn is no longer a potential host. The data presented here indicate that <50% of the late-season *H. zea* moths in the Bossier Parish of Louisiana had fed on cotton or soybean when they were larvae.

At least three hypotheses regarding *H. zea* biology are consistent with the Louisiana data presented here. The first is that the C_4 plants that produced these moths are local weeds or part of the natural local flora. The second hypothesis is that captured moths with a carbon isotope signature of C_4 plants, developed as larvae north of Louisiana where corn matures much later in the season. The third hypothesis is that local larvae that fed on corn or another C_4 plant earlier in the season had prolonged development or aestival pupal diapause and therefore emerged in late season.

The third hypothesis appears unlikely to hold based on a study conducted by Fye (33), in which only 2 of 129 *H. zea* larvae that hatched in the second half of July took 6 wk or more to mature. Furthermore, although larvae have been observed to develop
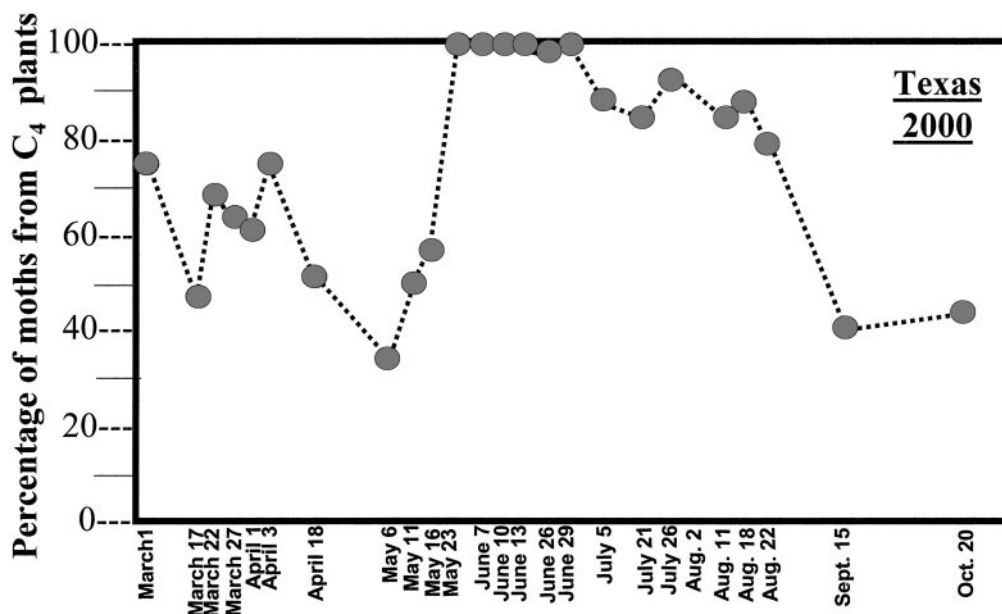
Fig. 4. Percentage of moths in College Station, TX, in 2000 with carbon isotope ratios indicative of having fed on a C_4 plant as larvae. On March 1, March 27, and April 3, 24 moths were tested. On all other dates, 25 moths were tested.

slowly on senescent corn (F.G. and J. R. Bradley, unpublished results), their numbers are limited, and the groups of adults produced should be clearly distinguishable due to small size. To distinguish between the first two hypotheses, we needed an area where there were few potential local crop and noncrop C_4 hosts of *H. zea*. The College Station area of Texas is such a place. Because of low rainfall late in the season, there is little suitable host plant material for *H. zea* larvae other than irrigated cotton. We hypothesized that in such an area there would be a lower proportion of moths with C_4 carbon isotope signatures late in the season compared with the proportion found in Louisiana. Our analysis of the late-season moths from this area over a 4-yr period indicates that at certain times, up to 75% of the moths could be coming from cotton, and that sometimes 0% of the moths can be coming from cotton. Because each sample analyzed included only 19–32 moths, the precision of each value is low. However, the average percentage of moths from C_3 hosts for all collection dates after August 1 for all 4 yr is 40.4%.

On the basis of our data, the hypothesis with strongest support is that late-season moths are migrating from more northern locations. We cannot conclusively state that this is the case based only on our study, but there are a number of other observations that also support this hypothesis. There is indirect evidence of *H. zea* and other noctuids moving south at this time of the year (34, 35). Pheromone and light trap data from northern areas such as Wisconsin and Iowa indicate that large numbers of moths are emerging in early September (J. Wedberg and R. Hellmich, unpublished data). Furthermore, ≈50% of pupae collected from corn in Wisconsin and in Pennsylvania in the first week of September did not diapause (F.G., J. Wedberg, and S. Fleisher, unpublished data) and are therefore available for migration. Finally, field studies have shown infestations of ≈16,000 large *H. zea* larvae per hectare of corn in the Midwest (e.g., ref. 36). Given the huge acreage of corn in the Midwest and the low incidence of diapause, successful southward migration of even a small fraction of the available *H. zea* moths would be important. That in some years higher numbers of moths are captured in Texas and Louisiana in September and October than at any other time of the year also supports the hypothesis of north to south migration.

A number of other rather simple studies would be useful to further test the hypothesis of north to south migration. First, further study of the potential existence of a common alternate C_4 host of *H. zea* in Louisiana and Texas in late summer should be pursued. Although extended summer pupal phases are not expected in *H. zea* based on insectary studies, detailed field experiments are warranted. As corn ears dry, they become unsuitable for larval development, but we do not have a quantitative estimate of the number of late instar larvae that complete development on these desiccated ears. A relevant ongoing study of moth weight and wing length indicates that moths with C_4 isotope profiles are larger than those with C_3 profiles even in late summer (T.L.R. and F.G., unpublished data). This would not be expected if C_4 moths had developed on marginally suitable drying corn ears.

Another important question raised by our data may be more difficult to answer: How much do late-season moths actually contribute to the population of *H. zea* in the following spring? In Texas, there appear to be few *H. zea* hosts available in September and October. If all of the eggs produced by late-season moths perished, they would contribute nothing in terms of resistance management. It could be that some of the captured moths are still en route toward tropical areas of Mexico, but this will be difficult to quantify. Our data from the 2000 season are of relevance here; they indicate that ≈63% of the moths flying during March and April in Texas did not develop on cotton. We cannot determine from our data whether the moths were migrants from Mexico, but there is compelling evidence from other studies that moths from Mexico are migrating to the U.S. in the spring (34, 37, 38).

We can conclude from our data that in midsummer, when corn is very suitable, <10% of *H. zea* are developing on cotton, and that in the later part of the season the proportion of *H. zea* moths produced in cotton and soybean is on average <50%, even in the College Station, TX area. Corn is most likely serving as the predominant alternate C_4 host for *H. zea*. Currently, <25% of U.S. corn produces Bt toxin. If, in the future, most field corn planted in the northern and southern U.S. remains in non-Bt-producing varieties, it could serve as a major *H. zea* refuge. Maintaining the current limit of 50% non-Bt corn in cotton-growing areas therefore seems appropriate for maintaining the

long-term utility of Bt cotton. Unless new evidence demonstrates that successful southward migration of *H. zea* is unlikely, it would be logical for the limit on Bt corn in the corn belt to be similar to that in cotton-growing areas. Currently there is little Bt corn grown in Mexico, but if this situation changes, another refuge for *H. zea* could be lost.

The 5% unsprayed/20% sprayed cotton refuge mandated by the EPA (www.epa.gov/pesticides/biopesticides/otherdocs/bt_cotton_refuge_2001.htm) is very relevant to resistance management for *Heliothis virescens* and *Pectinophora gossypiella*, which appear to have few other alternate host refuges. However, the data reported here indicate that for *H. zea* the current refuge in non-Bt corn is probably more critical to resistance management than the relatively small cotton refuge, and this corn refuge should be maintained.

In China and India, *Helicoverpa armigera* (Hubner) is a serious pest of cotton, and like *H. zea*, it is polyphagous and feeds on corn. In at least some areas of China, adoption of Bt cotton is very high, and non-Bt cotton refuges are not planted (39). Bt corn has not been commercialized in either country. Preliminary data demon-strate that *H. armigera* individuals reared on cotton and soybean in China have distinctly different carbon isotope profiles than *H. armigera* individuals reared on corn (K. Wu and F.G., unpublished data). Therefore, the isotope analysis technique used in the present study of *H. zea* could be very useful in determining whether non-Bt corn is serving as a useful refuge for *H. armigera* in China and India. Because carbon isotope ratios vary even within the C_3 and C_4 groupings of plants, in the future it may be possible to use stable isotope ratios to assess host use of herbivores that feed only on plants with C_3 or with C_4 physiology.

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